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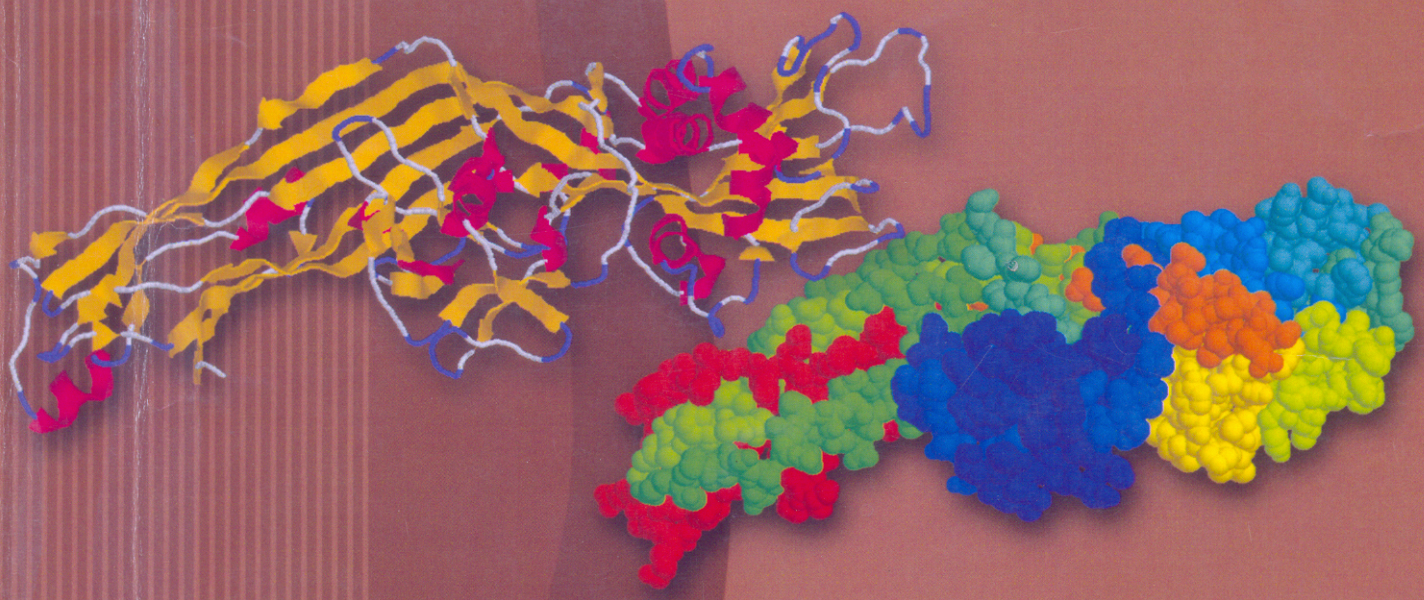

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IMMUNE FUNCTIONS IN CRUSTACEANS

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Introduction

The immune system of crustaceans has been studied in detail in only a limited number of species. There are some immune response systems that have been investigated intensively, such as the prophenoloxidase activating system (proPO), clotting, and phagocytosis. These investigations have led to unifying concepts based on molecular data from a variety of species. However, a comprehensive mechanistic approach to unravel the defense system of invertebrates is only possible when complete genomes, expressed genes and suitable disease challenge models are available. The crustaceans belong to the Arthropoda, which includes the insects. Genomes of two representatives of the latter namely the fruit fly (*Drosophila melanogaster*) and the mosquito (*Anopheles gambiae)* has been completely sequenced. The availability of the complete genome now allows a genome wide analysis of the immune responses in these insect species using standardized pathogen challenges. Expression profiling of immune responses in insects may be instrumental in unraveling more expediently the immune functions in crustaceans, especially of *Penaeus monodon,* as it has been suggested that this species is closely related to insects.

Identification of immune response genes in *Drosophila* and *Anopheles* has been performed in several ways. One approach uses cell lines, which are treated with LPS. RNA is extracted from the treated and untreated cell cultures at different time points. RNA is converted into cDNA and at the same time labeled with fluorescent dyes. The labeled cDNA and at the same time labeled with fluorescent dyes. The labeled cDNA pools are hybridized with a micro-array, containing all expressed genes of the organism used in the experiment. After hybridization, the micro arrays are washed and the relative fluorescence of each gene is determined. In this way gene expression can be determined as unaffected, up regulated or down regulated compared to the control treatment. The other makes use of *in vivo* challenges with pathogenic micro organisms, such as Gram positive, Gram-negative bacteria or a fungus. These experiments have lead to the identification of different immune genes involved in the responses to different pathogen challenges.

Expression profiling after challenge with gram-positive and gram-negative bacteria, and fungi identified two major pathways: Toll and Immunodeficiency (Imd). These pathways in insects comprise four steps 1. recognition, 2. regulation, 3. intracellular signal transduction, and 4. cellular and humoral responses. These pathways, including the cellular and humoral immune responses have been reviewed in detail. The first step involves Pattern Recognition Receptors (PRR), which have been identified throughout the animal kingdom and play an important role as they discriminate between self and infectious non-self. These receptors recognize unique conserved molecular patterns, such as lipopolysaccharides and peptidoglycans, found only in bacterial and fungal pathogens. The regulation of the recognition is facilitated and controlled mainly by activating and inhibitory CLIP domain serine protease. Intra-cellular signal transduction is highly conserved and comprises protein interactions similar to the NFKB signal pathway found in vertebrates. The protein modifications and interactions will eventually lead to the release of transcription factors, which are translocated to the nucleus and drive the expression of

immune response genes. Depending on the pathway used the response will either be the production of antimicrobial or antifungal peptides humoral response or activation of hemocytes (cellular response). However, to some degree, there is crosstalk between the two pathways.

Haemocytes

Crustacea have an open circulatory system with no equivalents to vertebrate red blood cells, but analogues of the white blood cells appear to exist. Crustacean haemocytes play an important role in the host immune responses including self/non-self recognition, phagocytosis, encapsulation, melanisation, cytotoxicity and cell-cell communication. The invertebrate blood cells collectively called as haemocytes or ceolomocytes were broadly classified in general, into progenitor cells, that are small, ranging from 4-10 µm in diameter with high nuclear cytoplasmic ratio; phagocytic cells, whose main function is phagocytosis of non-self material invading the host; homostatic cells, that participate in blood coagulation and wound sealing; Nutritive cells, that play an important role in nutrition and Pigmented cells, that are assumed to have respiratory pigments like haemocyanin. Percoll density gradient haemocyte population separation techniques followed by Soderhall and Smith (1983) in some decapod crustaceans paved the way for precise classification and characterization of crustacean haemocytes based on their morphological criteria into Granular, Semigranular and Hyaline cells. Similar classifications were made in urochordates, echinodermates, molluscs.

Martin and Graves (1985) studied fine structure and classification of penaeid shrimp haemocytes showing the morphology of hyaline cells with absence of any granules, although cytoplasmic inclusions of unknown origin was noticed. Similar findings were documented in palaemonid prawns by Tsing *et al.*, (1989). The hyaline cells of crustaceans were shown to have properties that readily attach and spread extensively on glass surfaces and phagocytose.

The semigranular cells contain a number of small granules, which upon activation lyse rapidly and release their contents *in vitro*. They concluded that the semigranular cell is responsible for recognizing and responding to foreign molecules and particles by de-granulation and subsequently attaching and spreading on the foreign surface in crustacea. Further the de-granulation response of semigranular cells could be induced by microbial lipopolysaccharides and β-1,3-glucan. Besides this, the encapsulation reaction was also found to be engaged by the semigranular cells.

The granular cell is filled with fairly large granules and its main function seems to be a repository for the prophenoloxidase activating system in freshwater crayfish,. The granular cell can be triggered to undergo exocytosis and release of the proPO system by two endogenous proteins, namely, the 76 kDa factor and the β-1, 3-glucan binding protein. Hose *et al.*, (1987) studied the shrimp haemocytes cytologically to show granular and semigranular cells to contain granules of proPO and peroxinectin. Division of circulatory haemocytes in *Penaeus paelensis, Macrobrachium rosenbergii, M. acanthurus* was observed. Thus, the new haemocytes need to be compensatory and proportionally produced and it is commonly believed that haemocytes are released continuously, although at varying rates, from a specialized haematopoietic tissue identified in several crustaceans like penaeid shrimp, *Sycionia ingentis* and lobster, *Homarus americanus*. In many crustacea, the sheet like haematopoietic tissue is situated on and covers the dorsal and dorso-ventral sides of the stomach and is surrounded by connective tissue.

Most invertebrate groups have a variety of fixed haemocytes that may play some role in the humoral and cellular defense mechanisms. Those include reticulum cells, podocytes, pore cells, and sinus lining cells of gastropod molluscs and nephrocytes of crustaceans. Crustacean nephrocytes have been shown to be involved in the sequestration of soluble and small particulate materials from the blood. Following injection with bacteria, the gill nephrocytes become filled with debris and there was a marked increase in the number of pinocytotic vesicles.

Phagocytosis

Phagocytosis is the most common of the cellular immune mechanisms and together with natural humoral factors, undoubtedly forms the first line of defence, once the physico-chemical barriers have been breached and hemolymph coagulation and blood cell clotting have been circumvented. Crustaceans have been shown to be capable of overcoming infection by the clearance of foreign materials from the hemolymph. This was thought due to phagocytosis by the circulatory cells, followed by accumulation of the haemocytes in the capillary and lacunar networks, where a system of fixed phagocytes further participate in the degradation of infective particles. Regarding the process of phagocytosis to remove a wide range of bacteria, virus, parasite and fungi, chemotaxis, attachment, ingestion and killing stages can be identified in invertebrates. Several studies have been done on this phenomenon in different crustacea. Phagocytosis by separated haemocyte populations *in vitro* has been examined in the freshwater crayfish, in which the hyaline and the semigranular cells were phagocytic, where as in case of crabs it is restricted to the hyaline cells. In shrimp it is accompanied by the granulocytes, primarily the small granular haemocytes.

Using non-separated haemocytes in crustaceans, phagocytic rates ranging from 1-2% to 28% have been recorded. Soderhall *et al.*, (1986) reported phagocytosis from the isolated haemocytes of *Carcinus maenas*. In a detailed study of phagocytosis in the freshwater crayfish, *Parachaeraps bicarinatus*, it was found that *in vitro* phagocytosis of erythrocytes by haemocytes of the cray fish, required specific opsonins. These opsonins appeared to be haemagglutinins, which enhanced adhesion of erythrocytes to haemocytes.. A variety of vaccines from other gram-negative bacteria or Lipopolysaccharide endotoxins also increased resistance to Pseudomonas infection. The immunity was ascribed to a change in activity of phagocytic cells.

Efficient uptake of bacteria was dependant upon factor(s) present in the haemolymph. Smith and Soderhall (1983b) treated haemocyte monolayer with β-1, 3-glucan, a trigger of proPO system activation, to show five to seven times higher degree of phagocytosis than untreated control monolayer. This particular finding shows evidence for the involvement of proPO system in phagocytosis. Tyson and Jenkin (1973) have shown that removal of bacteria form the circulation of the freshwater crayfish *P. bicarinatus* follows an exponential curve and is dependant on the presence of opsonic factors in the haemolymph. Later on Soderhall *et al.*, (1986) using isolated hyaline cells from *C. maenas*, showed that the phagocytic rate increased three times by opsonic factors present in the haemocyte lysate. Opsonic factors in crustaceans are thought to be either the products of proPO activation or agglutinins, specifically lectins. These opsonic factors were only produced if the proPO system in the haemocyte lysate induced to its active form. Phenoloxidase as such appeared to lack opsonic properties. The release of proPO granules is thought to be the initial event in recognition of the non-self particle and activation of phagocytes.

The phagocytic defence reactions of the shore crab, *C. maenas* were studied following injections of the bacteria, *Bacillus cereus* and *Morexella* sp., by histological and ultrastructural examination of the gills, heart and hepatopancreas. The *in vitro* phagocytosis of giant freshwater prawn, *M. rosenbergii* were studied following administration of Lipopolysaccharide (LPS) showed all the three types of haemocytes are able to phagocytose foreign particles.

During phagocytosis, particles or microorganisms are internalized into the cell, which later forms a digestive vacuole called the phagosome. The elimination of phagocytosed particles involves the release of degradative enzymes into the phagosome and the generation of Reactive Oxygen Intermediaries (ROIs) like O-2 subsequently releasing hydrogen peroxide (H2O2), hydroxyl radicals (OH-) and singlet oxygen (1O2) through the process of respiratory burst (Rodriguez and Moullac, 2000). Hydrogen peroxide can be converted to hypochlorous acid (HOCl-) via the myeloperoxidase (MPO)-H2O2-CL system, forming a potent antibacterial system. Bell and Smith (1993), showing the O-2 generation from hyaline cells using phorbol myristate acetate (PMA) as elicitor, gave the first evidence of ROIs production in crustacean haemocytes.

Nodule Formation

Nodule formation has been reported in most of the invertibrates including crustaceans, when microbial invasion is far in excess of the phagocytic capabilities of the host. When the haemocoel/coelom is invaded by numbers of microorganisms in excess of those that can be effectively cleared by phagocytosis along, nodule or cell clump formation occurs in many invertebrates, thus parasites being entrapped in masses of phagocytic or nonphagocytic blood cells which may or may not, depending on the animal involved, become melanised and effectively kill the parasites. The bacteria were found adhering outside to the granular haemocytes, which then aggregate to form cell clumps that subsequently enlarge by the addition of haemocytes, forming concentric flattened layers around a central, compact, often necrotic and sometimes melanised core.

Encapsulation

Haemocytes encapsulations by free circulating cells are stimulated to adhere to substratum and to each other, when a large parasite is encountered. Larger entities are encapsulated by haemocytes that attach and spread over them and eventually build up to several layers of cells. Thus sealed off from the circulation.

In addition to phagocytosis and nodule formation, invertebrate haemocytes are capable of immobilizing or killing the parasites, such as cestodes, trematodes, nematodes, parasitoides, fungi and large protozoans that are too large to be ingested by a single blood cell by surrounding them with multicellular sheaths. Person *et al.*, (1987b) have demonstrated in crayfish *Astacus leptodactylus* that, *in vitro* encapsulation was carried out by semigranular cells on a variety of foreign particles tested, regardless of their origin and surface charge and granular cells on fungal spores. Similarly Azad *et al.*, (1995) have studied that the most of the crustaceans semi-granular haemocytes are the first to react to a foreign particles or organism by encapsulation. However, the hyaline cells could not be noticed to be involved in the defense reaction. It has been suggested that the components produced in the process of activation of the prophenoloxidase system are involved in the encapsulation response in arthropods. Several groups have found that substances, which elicit proPO activation in arthropods, also enhance phagocytosis in *vitro* (Smith and Soderhal, 1983a; Ratcliffe *et al.*, 1984; Leonard *et al.*, 1985) and that a haemocyte lysate supernatant in which

Prophenolxidase system acts as a major recognition and defence pathway in crustaceans and insects. The melanisation reaction, which is a common response to parasite entry in vertebrates, especially arthropods, is due to the activity of an oxidoreductase, phenoloxidase (PO). The enzyme is a part of a complex system of proteinases, pattern recognition proteins and proteinase inhibitors constituting the so-called prophenoloxidase (proPO) system. The activation of proPO system results in the production of various proteins, including PO, which participate in melanisation around the parasite, coagulation, opsonisation of foreign materials and direct microbial killing. Furthermore, the proPO system has been proposed as invertebrate counterpart of the vertebrate complement system since it can be activated by β-1, 3-glucan and has got cascade reaction involving proteinases.

PO is a bifunctional copper containing terminal enzyme of the whole system, which catalyses both the O-hydroxylation of monophenols and the oxidation of phenols to quinones, thus converting tyrosine to dihydroxyphenylalanine (DOPA), as well as DOPA to DOPA-quinone, followed by several intermediate steps that lead to the synthesis of melanin, a brown pigment. A 30 kD PO has been purified and characterized in *Penaeus setiferus* by affinity chromatography. Further, a purified a 76 kD glycoprotein proenzyme, proPO, from the blood cells of the crayfish. However, the conversion of inactive proPO into active PO needs a serine proteinase, named the prophenoloxidase-activating enzyme (ppA), which has been isolated and purified from several arthropods, viz., *Bombyx mori* and crayfish.

As early as 1977, Unestam and Soderhall showed that PO activity could be strongly activated from its inactive from, proPO to active PO by β-1, 3-glucans from fungal cell walls. Ochiai and Ashida (1988) purified a β-1, 3-glucan-recognition protein that when coupled with ligand activates the proPO system in silkworm plasma. Similar observations were recorded in purified β-1, 3-glucan binding proteins of crayfish, *Pacifastacus leniusculus*. In both crustaceans and insects, the proPO system can be specifically activated by β-1, 3-glucan, which are surface components of fungal hyphae. Johansson and Soderhall (1985) concluded that in crayfish, *Pacifastacus leniusculus*, lipopolysaccharide (LPS) serves as an indicator of Gram-negative bacteria and β-1, 3-glucan as an indicator of fungi, that elicits both the exocytotic release of the proPO system from the semigranular cells and the subsequent biochemical activation of this system. It was also shown that microbial cell wall-derived LPS could also initiate the cascade in the crayfish, *Astacus astacus*, in *Procambarus clarkii*, induced by LPS, glycolipids, zymogen A and trypsin, by trypsin in *Carcinus maenas* and in *Pacifastacus leniusculus*. Aspan and Soderhall (1991) concluded that crayfish proPO could be converted to active form, the terminal enzyme in the proPO activating cascade by an apparent proteolytic cleavage, not only by a commercial proteinase, but also an endogenous serine type proteinase. The evidence that the proPO system generates non-self recognition factors in arthropods is confined to two studies with the crustaceans, *Astacus astacus* and *Carcinus maenas*. Smith and Soderhall (1983b) have suggested the attaching proteins of the proPO cascade are strong non-self signals for the haemocytes, causing them to degranulate and release previously cell-bound recognition factors into the haemolymph, where they are free to trigger activation of adjacent haemocytes.

Humoral Defence Factors

Hydrogen peroxide is scavenged by catalase enzyme to form water and oxygen and by peroxidase in the presence of reducing agent. In human neutrophils, myeloperoxidase is a heam containing protein present during phagocytosis, will form hypochlorous acid

(HOCl-) from hydrogen peroxide and chloride ions. HOCl will then destroy the ingested microbes. Eosinophilic peroxidase is a related protein involved in defence against larger parasites. Two peroxidase of this family have been found in Drosophila, where one of these, peroxidasin, has some peculiar properties characteristic of extra cellular matrix protein: The peroxidase domain is combined with six leucin-rich regions, four Ig loops, a thrombospondin/per collagen homology and an amphipatic α-helix. Another haem dependant peroxidase family is the cytochrome c-like peroxidase. Glutathione peroxidase and catalase are other type of enzymes that scavenge hydrogen peroxidase. Invertebrates, although lack immunoglobulins, have a range of factors that mediate agglutinating, lytic and antimicrobial activities against various biological agents, These factors may be naturally occurring and/or formed after pathogenic stimulation, but generally do not show the amnestic properties of the immunoglobulin as in case of vertebrates.

Lectins/Agglutinins/Haemagglutinins

Lectins/agglutinins are in general multivalent, or at least bivalent, carbohydrate-specific-binding ubiquitous proteins or glycoproteins usually without catalytic activity having various biological applications including cell sorting, cellular and humoral immune recognition mechanisms, immunomodulation and ability to agglutinate erythrocytes (haemagglutinin), bacteria and other normal and malignant cells. Ratanapo and Chulavatnatol (1992) reported the purification of monodin, a sialic acid- specific lectin from *P. monodon* was found to induce the agglutination of *Vibrio Vulnificus*, a major infective bacterium of the prawn. Haemolymph of the crab, *Scylla serrata* contains a lectin specific for N-glycolyl-neuraminic acid and was able to agglutinate when mammalian erythrocytes was used as a pathogen model. Bachere *et al.*, (1995) purified a lectin similar to monodin of *P. monodon* in the semi-granular haemocytes from *P. japonicus* using monoclonal antibodies.

Limulin, a sialic acid-specific lectin from the horseshoe crab, *Limulus polyphemus* (Chelicerata), reported to possess cytolytic-activity indicating an additional role for lectin invertebrates. Dyrynda *et al.*,(1995) reported cytotoxic activity in zoanthid, echinoderm , and eggs of opisthobranch molluscs and tunicate larvae.

In vitro studies have shown that invertebrate agglutinins facilitate phagocytosis by opsonisation of the non-self particles (McKay and Jenkin, 1970b). Arason (1996) has emphasized the possible role of lectins as non-self recognition molecules in vertebrate and invertebrate immunity. Results of Vazquez *et al.*, (1997) suggested that there is an active participation of sialic acid-specific lectin from the freshwater prawn *Macrobrachium rosenbergii* haemocytes in the recognition of non-self cells for active phagocytosis.

Haemolysins

Investigations on the lytic systems of invertebrates have concentrated chiefly on their ability to lyse vertebrate erythrocytes *in vitro*. Naturally occurring haemolysins have been found in a number of invertebrates, including molluscs and crustaceans.

Lysozyme-like activity

Primary immune response in crustaceans is non-specific cellular immunity. Haemocytes play a crucial role in this immune response because of their participation in phagocytosis, encapsulation, nodule formation, and cytotoxic mediation. Phagocytosis is the most common cellular defence reaction and in combination with humoral components, it consists the first line of defense against parasites or other intruders that evade the physicochemical barrier of the cuticle. Franchini and Ottaviani (1990) have shown that

phagocytes from the Cray fish, *Procambarus clarki*, produce lysosomal enzymes, which efficiently degrade and remove foreign material. Haemocyte phagocytosis in a number of animal species has only been proved indirectly by the detection of phagocytosis related lysosomal enzymes such as α-naphthyl acetate esterase, β-glucuronidase, and acid phosphatase.

Among the known antimicrobial peptides secreted by insects cell lines, lysozymes hydrolysed the β-1, 4 glycosidic bond between N-acetyl muramic acid and N-acetyl glucosamine in the peptidoglycan layer of the bacteria cell envelop. In Gram-positive organisms such as *Micrococcus luteus* , the peptidoglycan layer is found at the surface of the cell and varies considerably among species . Among Gram-positive organisms, the cell wall of *M. luteus* is more susceptible to degradation by lysozyme than that of other bacteria with which it has been compared. A lysozyme enzyme was purified and its properties were documented in an insect *Hyalophora cecropia*. Brewers yeast is a source of nucleic acids and polysaccharides including glucan; β-1, 3-glucans have been recognized to effectively enhance immune function of many aquaculture species including tiger shrimp *P. monodon* and rainbow trout.

Antimicrobial Factors

Antimicrobial factors that contain peptides, proteinases, defensins, cecropins and other antibacterial compounds appear to be ubiquitous and multipotent components of the innate immune defense arsenal used by both prokaryotic and eukaryotic organisms. They may either bring about the killing/ lysis of bacteria (bactericidal or bacteriolytic factors) or may act as a general disinfectant in inhibiting the growth (bacteriostatic). Initially crayfish serum and haemolymph when tested against marine and terrestrial bacteria for the presence of bacteriocins, no bacterial killing was noticed. Later Nakamura *et al.,* (1988) purified and studied the structure of tachyplesin, a class of antimicrobial peptide from the haemocytes of the horseshoe crab *Tachypleus tridentatus* that inhibits the growth of both Gram-positive and Gram-negative bacteria at low concentrations and formed a complex with bacterial lipopolysaccharide. An antimicrobial chitin-binding protein from horseshoe crab has been purified and christened tachycitin that has got growth inhibitory action on both Gram-positive and Gram-negative bacteria The penaeid shrimps possess penaeidins, peptides that combine with a proline rich amino terminal domain and a carboxyl domain containing six cysteines engaged in three disulfide bridges.

Decapod crustaceans have the capability of rapidly clearing invading bacteria from their haemolymph. It has been reported that the Shore crab, Lobsters, and penaeid shrimps removing in excess of 75% of bacterial cells within 10 min. to 1 hr. after injection. Both humoral & cellular responses are thought to be involved in this clearance reaction. Crustaceans possess a wide variety of non-cellular factors that are both naturally occurring, inducible, bioactive molecules e.g. agglutinin, killing factors, Lysins, precipitins, and clotting agents . Some antibacterial factors have been found in Lobster plasma, such as bacteriocidin, which were perceived as being effective against a variety of bacteria in lobsters.

Other plasmic proteins

Apart from haemocyanin, the respiratory protein which is quantitatively the most important of the circulating protein in crustaceans. Other similar kinds of immunopotential proteins are also reported. A α2- macroglobulin (α2-M)-like protein has been identified in the shrimp using Western-blot assay in crayfish and purified by the shrimp-specific

monoclonal antibody (Mab) (41B12). Shrimp α-2M-like protein appears to be dimmer of 340kD. In addition to the plasma, this protein is localized in the membrane of all haemocytes types and in large vesicular inclusions of the hyaline and semi-granular cells.

Pattern Recognition Receptors

Several pattern recognition receptors (PRRs) have been identified in crustaceans, most of them related to β-glucan recognition proteins. In the freshwater crayfish *Pacifastacus leniusculus* a lipopolysaccharide- and β-1,3-glucan binding protein (LBGP) has been isolated and characterized. LGBP has binding activity for LPS and β-1,3 glucans but not for peptidoglycans of Gram -positive bacteria. Experiments using a polyclonal antibody to block LBPG indicated that this protein plays a role in the activation of the proPO system. In other penaeid shrimp a BGP and a LBGP have been reported. The BGP from *Penaus monodon* is not up regulated, whereas the LBGP from *Penaeus stylirostris* is up regulated after bacterial infection. Peptidoglycan recognition proteins and Toll receptors have not yet been found. However, We have recently isolated a partial sequence encoding a TIR domain in *Penaeus monodon* . The presence of this TIR domain suggests that a Toll receptor or intra cellular signal transduction molecules such as MyD88 are present. Besides the three major PRR groups other receptors like αα2-microglobulin, C-type lectins of penaeids and calcium independent lectins have been reported.

Regulation of Immune Responses

Modulation and amplification of extra cellular cascades occurs mainly by serine protease. These protease are active in a large number of biochemical processes and those that perform an immune function by cleaving a specific ligand that interacts with a receptor are difficult to identify. A cell adhesion protein, a mass like protein, form *Pacifastacus leniusculus* shows homology with serine protease, especially to the *Drosophila* Masquerade and may be such a a candidate serine protease involved in immune responses. After binding of this masquerade-like protein to micro organisms, a proteolytic enzyme cleaves this protein. The intact form of the masquerade-like protein has been extensively characterized and contains seven putative disulphide knotted motifs and is implicated in the immune response to a bacterial infection.

Effector mechanisms

In the crustacean cellular response three groups of cells can be recognized based on morphological differences: the first are hyaline cells, which phagocytes invading organisms. The second group is called the semigranualr group, which have a much broader function, encapsulation, (limited) pahgocytosis, storage and release of proteins of the proPO system, and cytotoxicity. The latter two functions are also found in the third group, the granular cells. The first antimicrobial peptide characterized in crustaceans showed a high identity with bactenecin-7. The next group of antimicrobial peptides identified was the penaeidin family from the Pacific white shrimp *Litopenaeus vannamei*. Although this family shows similarities to the insect praline-rich antimicrobial peptides, they do not have a strong activity against gram negative bacteria. Callinectin, isolated form the hemocytes of the blue crab *Callinectes sapidus*, demonstrated activity against *E. coli* I and possessed a praline rich domain. The peptide does not show significant homology with any known peptide. Crustin, a new family of antimicrobial peptides, also show no homology with other known antibacterial peptides, but does display sequence identity with proteinase inhibitory proteins.

The prophenoloxidase (proPO) cascade-activating system is implicated in the immediate defense of shrimp against a variety of stimulatory conditions. This system has

been extensively studied. Phenoloxidase(PO; EC 1.14.18.1) is present in the hemolymph as an inactive pro-enzyme, proPO. Common chemical components of bacteria and fungi, such as β-1,3 glucans, are reacting with β-glucan binding protein (BGBP) and this complex induces degranulation and the activation of proPO system. This protein cascade is widely expressed and highly conserved in crustaceans. Proteins of the proPO system thus occupy a prominent position in non-self recognition, hemocyte communication and the production of melanin. Upon activation and degranulation of haemocytes, the inactive proPO is converted into the active phenoloxidase(PO) by prophenoloxidase activating enzyme(ppA). This quinines, and after several intermediate steps the formation of melanin. During this formation antimicrobial factors are formed. Melanin is a dark brown pigment that sequesters the pathogens, thus preventing their contact with the host. The melanization process is observed in response to foreign intruders in the haemocoel and during wound healing. An important factor that is associated with the proPO system is peroxinectin, which ws recently cloned for *P. monodon*. Peroxinectin displays cell- adhesion and peroxidase activity. Crayfish peroxinectin is synthesized in the hemocytes, stored in thesecretory granules in an inactive form, released in response to stimuli and activated outside the cells. Transmebrane receptors of the integrin family on the hemocytes play an important role in the cell adhesion function of peroxinectin. The cell adhesion can lead to attachment, spreading, phagocytosis, encapsulation, nodule formation and agglutination (aggregation), while the antimicrobial properties of the peroxidase activity of the protein might help to kill invading micro-organisms,

Conclusion

As a result of the molecular sequence information currently available on the expressed genes in insects, experiments have been performed adopting standardized challenge protocols either with bacteria or fungi to identify potential immune response genes. The problem with these experiments is that about 50%of the genes that are modulated after the pathogen challenge have an unknown function. Nevertheless these experiments have led to the identification of a large number of proteins involved in the immune response, notably those of the Toll and Imd pathway. Some of the modulation observed might be due to wounding alone in the case of a bacterial challenge where the bacteria are introduced by pricking the flies with a septic needle. This issue needs to be resolved. The fungal challenge is a more natural challenge as spores are simply coated onto the flies and this procedure does not require rupture of the cuticle. The fungus has the capability to penetrate the cuticle by itself. Given the relative close relationship between insects and crustaceans it is anticipated that the major immune signaling components and the full suit of effectors can be identified using the information from insects. However,.this only applies for anti-bacterial and anti-fungal responses, but not for the immune response to pathogenic viruses. As far as we can ascertain little attention has been paid to the immune response to viruses, neither in insects not in crustaceans. This could be due to the lack of appropriate viral challenge models. However, currently much research effort is devoted to the white spot syndrome virus(WSSV). This virus has become a pandemic within a relative short time span, and is particularly virulent in *Penaeus monodon*. The genome of the WSSV has been completely sequenced and turned out to be the largest animal viral genome of 292,967 base pairs with184 open reading frames. To investigate the aetiology of the virulence and the immune response to a viral infection in crustaceans, adopting a similar approach as used in flies, namely expression profiling using micro arrays, may provide clues which pathways, signaling cascades and effector mechanisms are involved.